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Linked Domain Architectures Allow for Specialization of Function in the FtsK/SpoIIIE ATPases of ESX Secretion Systems

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Abstract

Among protein secretion systems there are specialized ATPases that serve different functions such as substrate recognition, substrate unfolding, and assembly of the secretory machinery. ESX protein secretion systems require FtsK/SpoIIIE family ATPases but the specific function of these ATPases is poorly understood. The ATPases of ESX secretion systems have a unique domain architecture among proteins of the FtsK/SpoIIIE family. All well-studied FtsK family ATPases to date have one ATPase domain and oligomerize to form a functional molecular machine, most commonly a hexameric ring. In contrast, the ESX ATPases have three ATPase domains, either encoded by a single gene or by two operonic genes. It is currently unknown which of the ATPase domains is catalytically functional and whether each domain plays the same or a different function. Here we focus on the ATPases of two ESX systems, the ESX-1 system of *Mycobacterium tuberculosis* and the *yuk* system of *Bacillus subtilis*. We show that ATP hydrolysis by the ESX ATPase is required for secretion, suggesting that this enzyme at least partly fuels protein translocation. We further show that individual ATPase domains play distinct roles in substrate translocation and complex formation. Comparing the single chain and split ESX ATPases we reveal differences in the requirements of these unique secretory ATPases.

Keywords

secretory ATPases; mycobacterial protein secretion; Type VII Secretion System; ESX secretion; FtsK-like ATPases

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Introduction

The majority of protein export systems contain associated ATPases that supply energy for functions including assembly of the secretory apparatus and substrate translocation. FtsK/SpoIIIE family ATPases are conserved throughout bacteria and are involved in the translocation of DNA and proteins through membrane-spanning pores [1]. This translocation is important for cell division, sporulation, DNA conjugation, and other essential cell processes. FtsK/SpoIIIE family proteins are members of the larger FtsK-HerA superfamily of P-loop ATPases [1]. This superfamily includes VirB4-like ATPases of Type IV secretion systems, TrwB-like proteins involved in conjugal transfer of plasmids, and HerA family archeal helicases.

FtsK-HerA superfamily proteins share a characteristic molecular organization consisting of several N-terminal transmembrane segments followed by a cytoplasmic domain containing a single ATP-binding motif [2]. Because of the conservation and biological importance of these ATPases, numerous studies have investigated the structure and oligomeric state of these proteins. Structural analyses of FtsK, TwrB, and HerA have revealed that these proteins have a strong preference for forming ring-shaped hexamers [3-5], a preference extending throughout prokaryotic and eukaryotic organisms. For example, the single cytoplasmic ATPase domain from six FtsK monomers assemble into a homohexameric ring to create a pore with a central channel large enough to allow the passage of the double stranded DNA substrate [5]. The hexameric architecture of FtsK-HerA ATPases is a shared feature among many members of the larger AAA+ (ATPases Associated with various cellular Activities) and RecA protein families [6-8].

Recent work has revealed the presence of FtsK/SpoIIIE family ATPases in ESX secretion systems [9, 10]. ESX secretion systems (also referred to as Wss for WXG100 secretion system or Type VII in some instances) are a novel class of bacterial secretion systems [10-13]. The first identified ESX system was the ESX-1 secretion system of the human pathogen *Mycobacterium tuberculosis* [9, 14-16], and it has been shown that ESX-1 is a critical mediator of *M. tuberculosis* virulence [17, 18]. Subsequent genome sequence analyses have demonstrated a wide distribution of ESX systems throughout actinobacteria, firmicutes, chloroflexi, and several proteobacteria [9, 11, 16]. Within genetic loci encoding for ESX systems, two proteins are conserved among all species: an ATPase with multiple FtsK/SpoIIIE-like domains and a small substrate protein homologous to the secreted virulence factor EsxA of *M. tuberculosis* [9]. The molecular function and mechanisms of action of these systems remain poorly understood.

There are several unique structural features of the secretory FtsK-like ESX ATPases. First, whereas canonical members of the FtsK/SpoIIIE protein family contain a single enzymatic domain, the FtsK/SpoIIIE family ATPases of ESX secretion systems are predicted to contain two or three ATPase domains linked within a single polypeptide [9, 10]. Another striking feature of these FtsK/SpoIIIE family ATPases is the architecture of the genes encoding these proteins. In most ESX systems, the multidomain ATPase is encoded for by a single gene. For example, the gene *yukBA* of the *Bacillus subtilis yuk* secretion system encodes one protein with three predicted ATPase domains (Fig. 1a). However, an alternative protein

architecture is found in some ESX systems. For example, in the ESX-1 system of *M. tuberculosis*, the open reading frame encoding the ATPase has been split into two genes, *eccCa* and *eccCb*, containing one and two ATPase domains respectively (Fig. 1a) [16].

As suggested by the conservation of FtsK/SpoIIIE family ATPases among ESX systems, these ATPases are integral components of these secretion systems. Studies of ESX secretion systems from multiple organisms have demonstrated that these ATPases are required for substrate secretion [17, 19-22]. In mycobacteria both proteins in the split ESX ATPase, EccCa and EccCb, are required for secretion but only the EccCb ATPase interacts with the EsxB substrate [23]. In *Staphylococcus aureus* there is a linked ESX ATPase EssC in which the second and the third domains are dispensable for secretion [19]. Therefore, it is unclear whether each of the ATPase domains within these proteins is required for function, and if so, whether the multiple active sites contribute equally to function. Moreover, it is unknown how the split protein architecture of these ATPases reflects or affects protein function and assembly.

In this work, we address these questions by studying the ATPases of ESX systems representing two different ATPase protein architectures – single-chain and split protein variants. First, we make use of the model system that we have developed using the *yuk* secretion system of *B. subtilis* and analyze the single-chain ESX ATPase YukBA [22, 24]. We show that there are different functional requirements for the three ATPase domains of YukBA, where catalytic activity of only the most N-terminal ATPase domain is required for secretion. The nucleotide binding and enzymatic function of the other two ATPase domains are not required for substrate secretion. In contrast, in the split ESX ATPase in *M. tuberculosis*, nucleotide binding by each of the three ATPase domains is required for protein secretion. Together, our data suggest that the FtsK/SpoIIIE family ATPases of ESX secretion systems supply energy for protein translocation via ATP hydrolysis by the most N-terminal ATPase domain. Nucleotide binding by the second and third ATPase domains may be required for assembly or oligomerization of the functional apparatus, a requirement that becomes essential for ESX ATPases demonstrating a split protein architecture. This requirement may be alleviated in ESX systems in which the ATPase is a single polypeptide chain containing three linked ATPase domains.

Results

The ATPase domains of YukBA are differentially required for YukE secretion in B. subtilis

In the *B. subtilis* ESX secretion system, the FtsK/SpoIIIE family ATPase YukBA is required for the secretion of the EsxA homologue YukE [21, 22] (Fig. 2a). The Walker A motif (GXXXXGKT/S) is the conserved nucleotide binding site of P-loop NTPase domains [25, 26]. YukBA contains three ATPase domains, each containing a Walker A motif with a conserved lysine (K) residue at amino acids K688, K1016, and K1299 (Fig. 1b). We sought to determine the requirement for each of the ATPase domains within YukBA in *yuk*-mediated secretion. We inactivated each ATPase domain of YukBA by mutagenizing the conserved lysine residues to an alanine (A), both individually and in combination. This mutation prevents ATP binding in other systems, thereby disrupting the enzymatic activity of the ATPase domain [27, 28].

We tested the ability of each ATPase mutant protein to complement the *B. subtilis* Δ*yukBA* strain for YukE secretion. As seen in Fig. 2, the Δ*yukBA* deletion strain failed to secrete YukE while introduction of a wild-type copy of *yukBA* into Δ*yukBA* partially restored YukE secretion. Mutating the most N-terminal ATPase domain of YukBA^{K688A}, completely abolished YukE secretion (Fig. 2a). Moreover, any YukBA mutant containing the K688A mutation (YukBA^{K688A,K1016A}, YukBA^{K688A,K1299A}, or YukBA^{K688A,K1016A,K1299A}) failed to complement Δ*yukBA* for secretion. This indicates that the first ATPase domain is fully required for YukE secretion.

In contrast, production of YukBA proteins with mutations of the catalytic lysines in the second or third ATPase domains, YukBA^{K1016A} or YukBA^{K1299A}, complemented YukE secretion in the Δ*yukBA* strain (Fig. 2a). Likewise, production of YukBA^{K1016A,K1299A} restore YukE secretion to wild-type levels. Thus, neither YukBA^{K1016} nor YukBA^{K1299} are required for YukE secretion, individually or in combination.

To provide supporting evidence that the phenotypic consequence of the YukBA Walker A motif lysine to alanine mutations are specific to the loss of ATPase activity, as opposed to a less specific effect such as a global perturbation in enzyme structure, we also mutated each active site lysine of *yukBA* to a threonine (T). This amino acid substitution is also commonly used to eliminate the enzymatic activity of an ATPase [29, 30]. Consistent with our previous results, we found that mutating the most N-terminal ATPase domain of YukBA, YukBA^{K688T}, resulted in a loss of YukE secretion (Fig. 2b). The YukBA^{K1016T}, YukBA^{K1299T}, and YukBA^{K1016T,K1299T} mutant proteins were able to complement YukE secretion, as was seen with the corresponding lysine to alanine mutations. We also tested that all of the mutant YukBA proteins are produced in the cells to ensure that observed secretion changes are not due to protein stability or expression alterations (Fig. 2). In summary, we show that the three ATPase domains of YukBA are not functionally equivalent.

Walker A mutants of YukBA interfere with the functionality of the wild-type ATPase

The most N-terminal ATPase domain of YukBA is required for YukE secretion. We next assessed whether the YukBA ATPase mutant proteins have a dominant negative phenotype when expressed in the presence of a wild-type copy of the protein. Upon expression of *yukBA*^{K688A} in an otherwise wild-type strain, we found a greater than 80% reduction in YukE secretion (Fig. 3a). Likewise, when the K688A mutation is combined with the mutation of either other Walker A motif (*yukBA*^{K688A,K1016A}, *yukBA*^{K688A,K1299A}, or *yukBA*^{K688A,K1016A,K1299A}), we saw dramatically reduced YukE secretion as compared to a strain in which wild-type YukBA was heterologously expressed. In agreement with these findings, we found similar inhibition of secretion when K688 was mutated to a threonine, either individually or in combination with other Walker box mutations (Fig. 3b). Thus, a YukBA mutant protein that has been mutated at the first Walker A motif (YukBA^{K688}) appears to retain some ability to interact with and subsequently inhibit wild-type components of the translocon.

We then assessed the effects of expressing *yukBA*^{K1016A} or *yukBA*^{K1299A} in the presence of a wild-type secretion system. Although neither Walker A motif was required for secretion

individually or in combination, both mutants had a minor effect on YukE secretion when lysines were substituted to alanines or threonines (Fig. 3a-b). Overall, experiments expressing mutant copies of the YukBA ATPase in the presence of the wild-type protein showed that the mutant protein can interfere with functioning of the wild-type ATPase, likely indicating that the proteins oligomerize.

ATP hydrolysis by the first ATPase domain of YukBA is required for secretion

Disruption of the Walker A residues by mutation commonly eliminates binding of nucleotides to the active site of the target P-loop ATPase, which subsequently renders nucleotide hydrolysis impossible. The Walker B motif, another consensus sequence found in ATPases (hhhhDE), is responsible for nucleotide hydrolysis [25, 26, 31]. To separate the effects of nucleotide binding and hydrolysis we introduced mutations in the Walker B motifs of two of the ATPase domains in YukBA, where we could identify such motifs (Fig. 1b). As one can see from Fig. 1a, the second and third ATPase domains of YukBA do not have the glutamate (E) following the conserved aspartate (D). In case of the second domain there is a nearby DE pair following the aligned hhhhDN. Therefore, to test the effect of eliminating ATP hydrolysis on the *yuk* secretion, we mutated to alanine Walker B residue D794 in the first domain and the two putative Walker B residues in the second domain, D1114 and D1121.

Using a similar secretion assay, we assessed YukE secretion upon ectopic expression of the YukBA Walker B mutants in the Δ*yukBA* strain. We found that the Walker B motif mutants showed similar phenotypes to those observed when we mutated the Walker A motif in each ATPase domain (Fig. 4a). YukBA^{D749A}, the Walker B mutant of the most N-terminal ATPase domain, failed to restore secretion in the absence of the wild-type ATPase. In contrast, mutating either of the two putative Walker B domains of the second ATPase domain (residues D1114 and D1121) did not affect YukE secretion. This is consistent with our results that nucleotide binding to these domains is not required for secretion. Therefore, ATP hydrolysis by the first ATPase domain of YukBA is critical for secretory function. We further tested the effect of expressing each Walker B mutant in the context of the wild-type protein and observed that the Walker B mutation in the most N-terminal ATPase domain exhibited a dominant negative phenotype, abrogating YukE secretion (Fig. 4b). Combined, these results are consistent with the idea that catalytic activity of the ESX ATPase is required for efficient transport.

The YukBA ATPase is integral membrane protein

YukBA is predicted to be a 171 kDa integral membrane protein. We performed a fractionation assay to confirm that YukBA localizes to the membrane as predicted by sequence analyses (Fig. 1a). In this experiment, YukBA labeled with GFP was present in the membrane fraction and solubilized by Triton detergent but not by 1M NaCl (Fig. 5) which is consistent with the prediction that YukBA is an integral membrane protein. Therefore, similar to the mycobacterial ESX ATPases, YukBA localizes to bacterial membrane.

Each ATPase domain of the split ESX ATPase EccCa-EccCb is required for secretion in M. tuberculosis

While many of the FtsK/SpoIIIE family ATPases associated with ESX secretion systems show a protein architecture like that of *B. subtilis* YukBA (multiple ATPase domains linked within a single polypeptide), a subset of systems, including the ESX-1 secretion system of *M. tuberculosis*, represents an alternative protein architecture where the open reading frame encoding the ATPase has been split into two genes. It is unclear how this varied protein architecture may affect the enzymatic requirements for such systems. To address this question, we used the *M. tuberculosis* system to understand the individual domain requirements of the ESX-1 ATPase EccCa and EccCb in ESX-1-mediated secretion.

Through the study of transposon mutants, *M. tuberculosis eccCa* and *eccCb* were previously shown to be required for ESX-1-mediated secretion of the protein substrates EsxA and EsxB [17, 18]. Similarly, we found that transposon insertions in *eccCa* and *eccCb* disrupted secretion of three ESX-1 substrates: EsxA, EsxB, and EspA (Fig. 6). These defects could be complemented by the episomal expression of *eccCa-eccCb* in the *eccCa*::Tn mutant and *eccCb* alone in the *eccCb*::Tn strain.

EccCa contains a single Walker A motif (K485), while EccCb contains two predicted Walker A motifs (K90 and K382) (Fig. 1). We mutated the conserved lysine of each ATPase domain to an alanine and then tested the ability of the mutant proteins to complement ESX-1 secretion in the corresponding *eccCa*- or *eccCb*-null strains. In these studies, complementation of *eccCa*::Tn with the $eccCa^{K485A}$ mutant expressed with *eccCb* failed to restore secretion of EsxA, EsxB, and EspA (Fig. 6), though expression of the wild-type *eccCa* gene with *eccCb* restored secretion. Similarly, mutating the *eccCb* ATPase domains individually or in combination ($eccCb^{K90A}$, $eccCb^{K382A}$, or $eccCb^{K90A,K382A}$) disrupted secretion of EsxA, EsxB, and EspA. Thus, each ATPase domain of *eccCa* and *eccCb* is required for ESX-1 mediated secretion.

Some EccCa and EccCb ATPase mutants are dominant negative on ESX-1-mediated secretion

We next asked whether the expression of the Walker A mutants exerted a dominant negative phenotype on ESX-1-mediated secretion when expressed in a wild-type background. Ectopic expression of each individual domain mutant ($EccCa^{K485A}$, $EccCb^{K90A}$, or $EccCb^{K382A}$) blocked secretion of all of the ESX-1 substrates in a dominant negative fashion (Fig. 7). The inhibition of secretion was not simply due to overexpression of EccCa or EccCb, which could in theory dilute away required interacting proteins, as overexpression of wild-type copies of these proteins did not affect ESX-1-mediated secretion. The dominant negative phenotype displayed by the three single EccCa-EccCb ATPase mutants suggests that these mutant proteins enter into secretory complexes and render the complex nonfunctional.

In contrast, ectopic expression of the double mutant protein $EccCb^{K90A,K382A}$ did not have a significant dominant negative effect on ESX-1-mediated secretion (Fig. 7). While the level of secretion in this strain was slightly reduced as compared to wild-type, the level of secretion was significantly greater than was seen with the expression of any individual

EccCa or EccCb ATPase mutant. The lack of a dominant negative effect did not reflect a lower expression level of the $EccCb^{K90A,K382A}$ protein as the $EccCb^{K90A,K382A}$ mutant protein was present at roughly the same abundance as the $EccCb^{K382A}$ protein, which had a dominant negative effect on secretion. These results suggest that EccCb residues K90 and K382 might be required for EccCb to form stable protein complexes.

Discussion

To date ESX ATPases are the only absolutely conserved components of the ESX secretory apparatus that are present in both firmicute-like and mycobacterial subtypes of these secretion systems [10]. These ATPases are required for substrate translocation in all tested ESX secretion systems [17, 19-22]. However, prior to this work, it was unknown whether nucleotide binding and hydrolysis within the three predicted ESX ATPase domains are required for substrate translocation. Additionally, it was unclear whether the domain requirements were the same in biological systems representing the two possible protein architectures –linked or split ATPase domains. Through mutagenic analysis of the ESX ATPases from two different secretion systems we established that ATP hydrolysis in only one of the ATPases is required for secretion. Nucleotide binding within the second and third domains in the linked ATPase is dispensable for substrate translocation. In contrast, the split ESX ATPases of mycobacterial ESX-1 system seem to rely on the nucleotide binding for assembly.

Protein translocation across cellular membranes requires energy that is usually supplied by the proton motive force and/or nucleotide triphosphate hydrolysis by ATPases and GTPases. For most of the known translocation pathways there is one or more associated secretory ATPase that, apart from energizing the protein passage through membrane *per se*, is also involved in substrate recognition, unfolding, and assembly of the secretion machinery itself. For example, in the Type IV secretion systems there are three associated ATPases – VirD4, VirB4, and VirB11. All three ATPases are implicated in powering substrate translocation by ATP hydrolysis while specializing differently in substrate binding and pilus biogenesis [32]. Multidomain ATPases of the ESX secretion systems were earlier hypothesized to fuel substrate translocation based on their absolute necessity for secretion [17, 19]. We tested this hypothesis by creating Walker B mutants of the separate ATPase domains. Our results clearly show that ATP hydrolysis is indeed required for ESX secretion. Moreover, it seems that catalytic activity of only the most N-terminal ATPase domain is essential for the secretory function.

Despite their common hexameric form, but consistent with their diversity of functions, there is striking variation in the patterns of ATP utilization and oligomerization within P-loop ATPases. The composition of a hexameric machine can either be homogenous, formed by six identical protein subunits, or heterogenous, where nonidentical proteins come together to form a hexamer. In homohexameric ATPases, nucleotide binding results in conformational changes in subunits that in turn determine the hydrolysis mechanism (for example, see [33, 34]). Several mechanisms have been proposed for the homohexameric ATPases in which all subunits become specialized due to allosteric effects and asymmetry (i.e. [35-38]). Heterohexameric ATPases represent a more extreme form of subunit specialization, where

active sites within a complex can be differentially required for enzyme function, serving active, regulatory, or structural roles. Two striking examples of heterohexameric ATPases are the heavy chain of the dynein motor protein and the minichromosome maintenance (MCM) complex. Dyneins are large microtubule-walking motors that contain six AAA+ ATPase domains concatenated within a single polypeptide. Of these six domains, four contain intact active sites that are able to bind and hydrolyze ATP while the other two domains have lost the conserved Walker A motif and are believed to play structural roles [39, 40]. These six ATPase domains with distinct functions come together to form an asymmetric ring-shaped oligomer [39, 41]. The MCM complex is a replicative helicase made up of six different proteins, Mcm2 through Mcm7 [42]. These six proteins, each containing a Walker A ATP binding motif, oligomerize to form a hexameric ring-shaped complex [43]. The six individual subunits can be divided into two functional subgroups where three subunits are responsible for ATP hydrolysis, while three subunits serve a regulatory function [44]. The unique linked protein domain structure of ESX ATPases and the demonstrated differences in functional requirement for each domain is most reminiscent of such heterohexameric ATPases. Based on our results, ESX ATPases represent a new striking case of domain specialization within the heteromultimeric ATPases in which one third of the ATPase fold is hydrolyzing ATP.

Whereas the enzymatic activity of the second two domains of the YukBA ATPase is not required for substrate translocation, we hypothesize that they may interact with the substrate as was shown for the corresponding mycobacterial domains [17, 23, 45]. It is also possible that these domains play a structural role in the assembly of the secretion apparatus.

Covalently linked multidomain ATPases are also known to form different assemblies. As described above, the dynein hexamer exists as a single layered asymmetric ring. In contrast, the two covalently linked ATPase domains of p97, NSF, and ClpB oligomerize into a two-tiered structure of stacked hexameric rings [46-50]. In such two-layered barrels one of the homohexamers is responsible for the ATP hydrolysis while the second homohexamer is only capable of nucleotide binding [51-53]. By expressing mutant versions of the ESX ATPases in the presence of the wild-type protein we observed that the mutant forms of the ATPases interfere with the functionality of the wild-type ATPases (Fig. 3a-b, 4b). Such interference as well as the dominant negative phenotypes of some substitutions is indicative of protein oligomerization. Moreover, these data are consistent with the expectation that FtsK-like ATPases tend to hexamerize to perform their functions and therefore it is likely that the YukBA ATPase oligomerizes. Mycobacterial EccCa and EccCb ATPases have previously been shown to interact [23] Interestingly, there are two possible ways to assemble the three ATPase domains into hexameric ring structures. One assembly implies formation of a dimer of trimers resulting in a single-layered ring, while the other assembly results in a three-layered barrel consisting of three homohexamers stacked on top of each other.

There is no known example of the assemblies of either sort. Data from *S. aureus* ESX ATPase EssC may favor the three-layered assembly as a functional unit of the ESX ATPases. EssC contains three linked ATPase domains and deletion of the second and third domains revealed that the last two domains are dispensable for a substrate secretion suggesting that the first ATPase domain might be capable of independent assembly [19].

Future studies will be necessary to determine which ESX ATPase oligomer represents a functional unit. The structure of the complex is of particular interest because the ESX systems are shown to translocate dimeric substrates and accommodate substrates of different sizes [24, 54, 55].

To date only one substrate has been identified for the *B. subtilis yuk* system, YukE [22], while the ESX-1 system has multiple protein substrates. For the mycobacterial ESX-1 ATPases it was shown that the EsxB substrate bound only to EccCb but not to EccCa under tested conditions [23] but the effect of the nucleotide interaction on secretion was not tested. Our analyses of the mycobacterial ESX-1 ATPases show that the EccCa and EccCb have the same specialized requirements for secretion of all three tested substrates – EsxA, EsxB, and EspA (Fig. 6-7). Consistent with the recent identification of widespread recognition motifs across ESX substrates [24, 54, 56], we conclude that the energy and ligand binding requirements are generalizable for different substrates of the mycobacterial ESX secretion system.

In our comparison of the linked and split ESX ATPases we found a striking difference in the nucleotide binding requirements for secretion. While the nucleotide interaction in the second and third domains of the linked YukBA ATPase is not required for substrate secretion, nucleotide binding to both of the EccCb ATPase domains is essential for secretory function. It is well documented that nucleotide interactions are required for the assembly of many multimeric ATPase motors. Therefore, we hypothesize that EccCa and EccCb assembly may also be regulated by the nucleotide interaction. Such a hypothesis may explain the result that the double Walker A mutant of EccCb does not exhibit a negative dominant phenotype while the single Walker A mutants do disrupt the function of the wild-type protein (Fig. 7). It is also interesting to consider that in the five ESX loci present in *M. tuberculosis* only two have split FtsK-like ATPases, the ESX-1 ATPases considered here, and the ESX-5 ATPases. Bioinformatics analyses of the mycobacterial genomes indicate that the ESX-4 locus is likely ancestral and it contains a linked EccC4 ATPase [16]. Thus it is tempting to propose that the splitting of the multidomain ATPases might have created an additional regulated assembly step that allows mycobacterial species to tune specificity and regulation of secreting the multitude of ESX-dependent substrates.

Materials and Methods

Strain construction

All *M. tuberculosis* strains were derived from the wild-type strain H37Rv. All *B. subtilis* strains were derived from the prototrophic strain PY79 [57]. General methods for molecular cloning and strain construction of *B. subtilis* strains were performed according to published protocols [58]. Chromosomal DNA isolated from *M. tuberculosis* H37Rv or *B. subtilis* PY79 was used as a template for all PCR amplification. Introduction of DNA into *B. subtilis* PY79 derivatives was conducted by transformation [59]. To generate *M. tuberculosis* complementing constructs, the genomic region encoding *eccCa*, *eccCb*, or *eccCa-eccCb* in tandem (including the native intergenic region), was amplified from H37Rv genomic DNA. Forward primers added a ribosomal binding site (GAAGGAGATATACAT) upstream of each start codon, and the reverse oligo of *eccCb* added a C-terminal Flag-tag (protein

sequence: DYKDDDDK). A second round of PCR added *att* sites for Gateway recombination of the product and the PCR products were recombined into a Gateway donor vector (pDONR, Invitrogen, Carlsbad, CA) and transferred to an episomal expression vector (pTETSG), which allows for regulated gene expression under the control of a tetracycline inducible mycobacterial promoter [60]. The resulting pTETSG constructs or an empty vector were transformed into wild-type H37Rv, *eccCa*::Tn, and *eccCb*::Tn strains. These strains were maintained under selection with 50 μg/ml Hygromycin (Roche, Mannheim, Germany) to prevent plasmid loss. Site directed mutagenesis of *eccCa* and *eccCb* expression vectors was performed to construct each Walker A mutant. The bacterial strains and plasmids used in this study are listed in Tables 1-4. All constructs were confirmed by sequencing.

Media and growth conditions

For general propagation, *M. tuberculosis* strains were maintained at 37°C in Middlebrook 7H9 broth medium supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC, Difco), 0.2% glycerol, and 0.05% Tween 80 or Middlebrook 7H10 agar containing 10% OADC, 0.5% glycerol, and 0.05% Tween 80. When appropriate, 50 μg mL^{-1} hygromycin was included in the growth medium. Where indicated in methods below, strains were cultured in Sauton's broth medium supplemented with 0.05% Tween 80 instead of 7H9 medium because the large quantity of albumin in 7H9 medium confounded analysis of secreted bacterial proteins.

B. subtilis strains were maintained at 37°C in lysogeny broth (LB) medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl) or on LB plates containing 1.5% Bacto agar. When appropriate, antibiotics were included in the growth medium as follows: 100 μg mL^{-1} spectinomycin, 5 μg mL^{-1} chloramphenicol, 5 μg mL^{-1} kanamycin, and 1 μg mL^{-1} erythromycin plus 25 μg mL^{-1} lincomycin (mls).

Preparation of cell lysates and culture filtrates

For analysis of protein expression and secretion by *M. tuberculosis*, bacterial cultures grown to mid-log phase were normalized to an OD_{600} of 0.3 in fresh 7H9 medium containing 100 ng mL^{-1} anhydrotetracycline (AT, Spectrum Chemicals), when appropriate, to induce protein expression. Cultures were grown overnight at 37°C to allow 24 hours for the induction of gene expression of the complementing construct prior to beginning culture supernatant collections. After 24 hours, cultures were pelleted, washed, resuspended in Sauton's medium supplemented with 0.05% Tween 80 and AT and grown for 48 hours at 37°C. Cell pellets were collected by centrifugation, resuspended in protein extraction buffer (50 mM Trism·HCL pH 7.5, 5 mM EDTA, protease inhibitor cocktail (Complete Mini, EDTA-free tablets, Roche)) and disrupted by bead beating. SDS sample buffer (Novex 2x Tricine SDS sample buffer, Invitrogen) was added and samples were heated at 95°C for 20 minutes before removal from the biosafety level 3 facility. Protease inhibitor cocktail was added to the culture supernatants, which were then sterilized by double filtration through 0.2 μM filters. The culture supernatants were concentrated by precipitation with 10% trichloroacetic acid (TCA), resuspended in SDS sample buffer and heated to 95°C for 20 minutes.

For analysis of protein expression and secretion by *B. subtilis* strains, mid-log bacterial cultures were started from a single colony, back diluted and normalized to an OD_{600} of 0.02 in LB medium, and grown 2 hours at 37°C, at which time 100 μM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to induce protein expression; cultures were grown an additional 2 hours. Cell pellets were collected by centrifugation, resuspended in lysis buffer (20 mM Tris·HCl pH 7.5, 10 mM EDTA, 1 mM PMSF, 10 μg mL^{-1} DNase I, 100 μg mL^{-1} RNaseA, 1 mg mL^{-1} lysozyme) and incubated at 37°C for 10 minutes. SDS sample buffer (Novex 2x Tricine SDS sample buffer, Invitrogen) was added and samples were heated to 95°C for 15 minutes. Culture supernatants were filtered though a 0.2 μM filter to remove unlysed cells. Following the addition of a protease inhibitor cocktail (Complete Mini, EDTA-free tablets, Roche), the supernatants were concentrated by precipitation with 10% TCA, resuspended in SDS sample buffer and heated to 95°C for 15 minutes.

SDS-PAGE and immunoblot analysis

Prior to analysis, samples were reduced with 100 mM dithiothreitol (DTT) for 1 hour at 37°C. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Bio-Rad) for immunoblot analysis. Membranes were probed with anti-EsxA (ESAT-6, 1:2,000, ab26246, Abcam), anti-EsxB (CFP-10, 1:1,000, ab45074, Abcam), anti-EspA (1:2,000, [61]), anti-Flag (1:5,000, F7425, Sigma), anti-YukE (polyclonal, 1:1,000, [22]) and anti-Myc (1:3,000, ab9106, Abcam) antibodies. Peroxidase-conjugated goat anti-rabbit (1:3,000, ab6721, Abcam) and goat anti-mouse (1:10,000, 32430, Thermo Scientific) secondary antibodies were detected by chemiluminescence using SuperSignal West Femto (Thermo Scientific). Antibodies to the cytosolic *E. coli* protein RNAP (1:1,000, WP023, Neoclone) or the cytosolic *B. subtilis* protein SigmaA (1:1,000,000 [62]) were used to ensure equal protein loading and as a lysis control for *M. tuberculosis* and *B. subtilis* immunoblots, respectively. Equal loading of culture supernatants was confirmed by visualizing the total protein loaded by Coomassie staining. Blots were imaged and densitometric quantitation of YukE, EsxA, EsxB, and EspA secretion was performed using a FluorChem FC2 gel documentation system (Alpha Innotech) and provided software.

Fractionation of B. subtilis lysates

YukBA labeled with GFP was ectopically expressed in *B. subtilis* upon IPTG induction. Whole cell lysates were prepared from cells grown in LB medium and harvested during mid-exponential phase. Soluble (S3) and insoluble (P3) protein fractions were separated by centrifugation at 3,000 × g. The S3 fraction was further separated by centrifugation at 100,000 × g to collect the soluble (S100) and insoluble (P100) fractions. The P100 fraction was divided into three equal volumes and incubated with either buffer, 1M NaCl, or 10% Triton detergent and then spun again at 100,000 × g to collect the soluble (S100) and insoluble (P100) fractions. The samples were analyzed by SDS-PAGE under reducing conditions and Western blot with anti-GFP antibodies.

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References

1. Iyer LM, Makarova KS, Koonin EV, Aravind L. Comparative genomics of the FtsK-HerA superfamily of pumping ATPases: implications for the origins of chromosome segregation, cell division and viral capsid packaging. Nucleic Acids Res. 2004; 32:5260–79. [PubMed: 15466593]
2. Moncalian G, Cabezon E, Alkorta I, Valle M, Moro F, Valpuesta JM, et al. Characterization of ATP and DNA binding activities of TrwB, the coupling protein essential in plasmid R388 conjugation. J Biol Chem. 1999; 274:36117–24. [PubMed: 10593894]
3. Gomis-Ruth FX, Moncalian G, Perez-Luque R, Gonzalez A, Cabezon E, de la Cruz F, et al. The bacterial conjugation protein TrwB resembles ring helicases and F1-ATPase. Nature. 2001; 409:637–41. [PubMed: 11214325]
4. Manzan A, Pfeiffer G, Hefferin ML, Lang CE, Carney JP, Hopfner KP. MlaA, a hexameric ATPase linked to the Mre11 complex in archaeal genomes. EMBO Rep. 2004; 5:54–9. [PubMed: 14710187]
5. Massey TH, Mercogliano CP, Yates J, Sherratt DJ, Lowe J. Double-stranded DNA translocation: structure and mechanism of hexameric FtsK. Mol Cell. 2006; 23:457–69. [PubMed: 16916635]
6. Neuwald AF, Aravind L, Spouge JL, Koonin EV. AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. Genome Res. 1999; 9:27–43. [PubMed: 9927482]
7. Singleton MR, Dillingham MS, Wigley DB. Structure and mechanism of helicases and nucleic acid translocases. Annu Rev Biochem. 2007; 76:23–50. [PubMed: 17506634]
8. Erzberger JP, Berger JM. Evolutionary relationships and structural mechanisms of AAA+ proteins. Annu Rev Biophys Biomol Struct. 2006; 35:93–114. [PubMed: 16689629]
9. Pallen MJ. The ESAT-6/WXG100 superfamily -- and a new Gram-positive secretion system? Trends Microbiol. 2002; 10:209–12. [PubMed: 11973144]
10. Abdallah AM, Gey van Pittius NC, Champion PA, Cox J, Luirink J, Vandenbroucke-Grauls CM, et al. Type VII secretion--mycobacteria show the way. Nat Rev Microbiol. 2007; 5:883–91. [PubMed: 17922044]
11. Sutcliffe IC. New insights into the distribution of WXG100 protein secretion systems. Antonie Van Leeuwenhoek. 2011; 99:127–31. [PubMed: 20852931]
12. Chagnot C, Zorgani MA, Astruc T, Desvaux M. Proteinaceous determinants of surface colonization in bacteria: bacterial adhesion and biofilm formation from a protein secretion perspective. Frontiers in microbiology. 2013; 4:303. [PubMed: 24133488]
13. Bitter W, Houben EN, Bottai D, Brodin P, Brown EJ, Cox JS, et al. Systematic genetic nomenclature for type VII secretion systems. PLoS Pathog. 2009; 5:e1000507. [PubMed: 19876390]
14. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature. 1998; 393:537–44. [PubMed: 9634230]
15. Tekaia F, Gordon SV, Garnier T, Brosch R, Barrell BG, Cole ST. Analysis of the proteome of Mycobacterium tuberculosis in silico. Tubercle and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease. 1999; 79:329–42.
16. Gey Van Pittius NC, Gamieldien J, Hide W, Brown GD, Siezen RJ, Beyers AD. The ESAT-6 gene cluster of Mycobacterium tuberculosis and other high G+C Gram-positive bacteria. Genome Biol. 2001; 2 RESEARCH0044.
17. Stanley SA, Raghavan S, Hwang WW, Cox JS. Acute infection and macrophage subversion by Mycobacterium tuberculosis require a specialized secretion system. Proc Natl Acad Sci U S A. 2003; 100:13001–6. [PubMed: 14557536]
18. Guinn KM, Hickey MJ, Mathur SK, Zakel KL, Grotzke JE, Lewinsohn DM, et al. Individual RD1-region genes are required for export of ESAT-6/CFP-10 and for virulence of Mycobacterium tuberculosis. Mol Microbiol. 2004; 51:359–70. [PubMed: 14756778]

19. Burts ML, Williams WA, DeBord K, Missiakas DM. EsxA and EsxB are secreted by an ESAT-6-like system that is required for the pathogenesis of Staphylococcus aureus infections. Proc Natl Acad Sci U S A. 2005; 102:1169–74. [PubMed: 15657139]
20. Akpe San Roman S, Facey PD, Fernandez-Martinez L, Rodriguez C, Vallin C, Del Sol R, et al. A heterodimer of EsxA and EsxB is involved in sporulation and is secreted by a type VII secretion system in Streptomyces coelicolor. Microbiology. 2010; 156:1719–29. [PubMed: 20223806]
21. Baptista C, Barreto HC, Sao-Jose C. High Levels of DegU-P Activate an Esat-6-Like Secretion System in Bacillus subtilis. PloS one. 2013; 8:e67840. [PubMed: 23861817]
22. Huppert LA, Ramsdell TL, Chase MR, Sarracino DA, Fortune SM, Burton BM. The ESX System in *Bacillus subtilis* Mediates Protein Secretion. PloS one. 2014 in production.
23. Champion PA, Stanley SA, Champion MM, Brown EJ, Cox JS. C-terminal signal sequence promotes virulence factor secretion in Mycobacterium tuberculosis. Science. 2006; 313:1632–6. [PubMed: 16973880]
24. Sysoeva TA, Zepeda-Rivera MA, Huppert LA, Burton BM. Dimer recognition and secretion by the ESX Secretion System in *Bacillus subtilis*. Proc Natl Acad Sci U S A. 2014 in press.
25. Walker JE, Saraste M, Runswick MJ, Gay NJ. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. 1982; 1:945–51. [PubMed: 6329717]
26. Saraste M, Sibbald PR, Wittinghofer A. The P-loop--a common motif in ATP- and GTP-binding proteins. Trends Biochem Sci. 1990; 15:430–4. [PubMed: 2126155]
27. Omote H, Maeda M, Futai M. Effects of mutations of conserved Lys-155 and Thr-156 residues in the phosphate-binding glycine-rich sequence of the F1-ATPase beta subunit of Escherichia coli. J Biol Chem. 1992; 267:20571–6. [PubMed: 1400377]
28. Stephens KM, Roush C, Nester E. Agrobacterium tumefaciens VirB11 protein requires a consensus nucleotide-binding site for function in virulence. J Bacteriol. 1995; 177:27–36. [PubMed: 7798144]
29. Seol JH, Baek SH, Kang MS, Ha DB, Chung CH. Distinctive roles of the two ATP-binding sites in ClpA, the ATPase component of protease Ti in Escherichia coli. J Biol Chem. 1995; 270:8087–92. [PubMed: 7713911]
30. Schirmer EC, Queitsch C, Kowal AS, Parsell DA, Lindquist S. The ATPase activity of Hsp104, effects of environmental conditions and mutations. J Biol Chem. 1998; 273:15546–52. [PubMed: 9624144]
31. Hanson PI, Whiteheart SW. AAA+ proteins: have engine, will work. Nature reviews Molecular cell biology. 2005; 6:519–29.
32. Trokter M, Felisberto-Rodrigues C, Christie PJ, Waksman G. Recent advances in the structural and molecular biology of type IV secretion systems. Curr Opin Struct Biol. 2014; 27C:16–23. [PubMed: 24709394]
33. Martin A, Baker TA, Sauer RT. Rebuilt AAA + motors reveal operating principles for ATP-fuelled machines. Nature. 2005; 437:1115–20. [PubMed: 16237435]
34. Gai D, Zhao R, Li D, Finkielstein CV, Chen XS. Mechanisms of conformational change for a replicative hexameric helicase of SV40 large tumor antigen. Cell. 2004; 119:47–60. [PubMed: 15454080]
35. Glynn SE, Martin A, Nager AR, Baker TA, Sauer RT. Structures of asymmetric ClpX hexamers reveal nucleotide-dependent motions in a AAA+ protein-unfolding machine. Cell. 2009; 139:744–56. [PubMed: 19914167]
36. Sysoeva TA, Chowdhury S, Guo L, Nixon BT. Nucleotide-induced asymmetry within ATPase activator ring drives sigma54-RNAP interaction and ATP hydrolysis. Genes & development. 2013; 27:2500–11. [PubMed: 24240239]
37. Itsathitphaisarn O, Wing RA, Eliason WK, Wang J, Steitz TA. The hexameric helicase DnaB adopts a nonplanar conformation during translocation. Cell. 2012; 151:267–77. [PubMed: 23022319]
38. Enemark EJ, Joshua-Tor L. Mechanism of DNA translocation in a replicative hexameric helicase. Nature. 2006; 442:270–5. [PubMed: 16855583]

39. Carter AP, Cho C, Jin L, Vale RD. Crystal structure of the dynein motor domain. Science. 2011; 331:1159–65. [PubMed: 21330489]
40. Vallee RB, Hook P. Autoinhibitory and other autoregulatory elements within the dynein motor domain. J Struct Biol. 2006; 156:175–81. [PubMed: 16647270]
41. Schmidt H, Gleave ES, Carter AP. Insights into dynein motor domain function from a 3.3-A crystal structure. Nature structural & molecular biology. 2012; 19:492–7. S1.
42. Adachi Y, Usukura J, Yanagida M. A globular complex formation by Nda1 and the other five members of the MCM protein family in fission yeast. Genes to cells : devoted to molecular & cellular mechanisms. 1997; 2:467–79. [PubMed: 9366552]
43. Bochman ML, Schwacha A. The Mcm complex: unwinding the mechanism of a replicative helicase. Microbiology and molecular biology reviews : MMBR. 2009; 73:652–83. [PubMed: 19946136]
44. Schwacha A, Bell SP. Interactions between two catalytically distinct MCM subgroups are essential for coordinated ATP hydrolysis and DNA replication. Mol Cell. 2001; 8:1093–104. [PubMed: 11741544]
45. DiGiuseppe Champion PA, Champion MM, Manzanillo P, Cox JS. ESX-1 secreted virulence factors are recognized by multiple cytosolic AAA ATPases in pathogenic mycobacteria. Mol Microbiol. 2009; 73:950–62. [PubMed: 19682254]
46. Furst J, Sutton RB, Chen J, Brunger AT, Grigorieff N. Electron cryomicroscopy structure of N-ethyl maleimide sensitive factor at 11 A resolution. EMBO J. 2003; 22:4365–74. [PubMed: 12941689]
47. Zhang X, Shaw A, Bates PA, Newman RH, Gowen B, Orlova E, et al. Structure of the AAA ATPase p97. Mol Cell. 2000; 6:1473–84. [PubMed: 11163219]
48. Haslberger T, Weibezahn J, Zahn R, Lee S, Tsai FT, Bukau B, et al. M domains couple the ClpB threading motor with the DnaK chaperone activity. Mol Cell. 2007; 25:247–60. [PubMed: 17244532]
49. Lee S, Sowa ME, Watanabe YH, Sigler PB, Chiu W, Yoshida M, et al. The structure of ClpB: a molecular chaperone that rescues proteins from an aggregated state. Cell. 2003; 115:229–40. [PubMed: 14567920]
50. Huyton T, Pye VE, Briggs LC, Flynn TC, Beuron F, Kondo H, et al. The crystal structure of murine p97/VCP at 3.6A. J Struct Biol. 2003; 144:337–48. [PubMed: 14643202]
51. May AP, Whiteheart SW, Weis WI. Unraveling the mechanism of the vesicle transport ATPase NSF, the N-ethylmaleimide-sensitive factor. J Biol Chem. 2001; 276:21991–4. [PubMed: 11301340]
52. Sumida M, Hong RM, Tagaya M. Role of two nucleotide-binding regions in an N-ethylmaleimide-sensitive factor involved in vesicle-mediated protein transport. J Biol Chem. 1994; 269:20636–41. [PubMed: 8051162]
53. Rouiller I, DeLaBarre B, May AP, Weis WI, Brunger AT, Milligan RA, et al. Conformational changes of the multifunction p97 AAA ATPase during its ATPase cycle. Nature structural biology. 2002; 9:950–7.
54. Daleke MH, Ummels R, Bawono P, Heringa J, Vandenbroucke-Grauls CM, Luirink J, et al. General secretion signal for the mycobacterial type VII secretion pathway. Proc Natl Acad Sci U S A. 2012; 109:11342–7. [PubMed: 22733768]
55. Garufi G, Butler E, Missiakas D. ESAT-6-like protein secretion in Bacillus anthracis. J Bacteriol. 2008; 190:7004–11. [PubMed: 18723613]
56. Poulsen C, Panjikar S, Holton SJ, Wilmanns M, Song YH. WXG100 protein superfamily consists of three subfamilies and exhibits an alpha-helical C-terminal conserved residue pattern. PloS one. 2014; 9:e89313. [PubMed: 24586681]
57. Youngman PJ, Perkins JB, Losick R. Genetic transposition and insertional mutagenesis in Bacillus subtilis with Streptococcus faecalis transposon Tn917. Proc Natl Acad Sci U S A. 1983; 80:2305–9. [PubMed: 6300908]
58. Sambrook, DW, JR. The condensed protocols from molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 2006.

59. Gryczan TJ, Contente S, Dubnau D. Characterization of Staphylococcus aureus plasmids introduced by transformation into Bacillus subtilis. J Bacteriol. 1978; 134:318–29. [PubMed: 418061]

60. Ehrt S, Guo XV, Hickey CM, Ryou M, Monteleone M, Riley LW, et al. Controlling gene expression in mycobacteria with anhydrotetracycline and Tet repressor. Nucleic Acids Res. 2005; 33:e21. [PubMed: 15687379]

61. Fortune SM, Jaeger A, Sarracino DA, Chase MR, Sassetti CM, Sherman DR, et al. Mutually dependent secretion of proteins required for mycobacterial virulence. Proc Natl Acad Sci U S A. 2005; 102:10676–81. [PubMed: 16030141]

62. Fujita M. Temporal and selective association of multiple sigma factors with RNA polymerase during sporulation in Bacillus subtilis. Genes to cells : devoted to molecular & cellular mechanisms. 2000; 5:79–88. [PubMed: 10672039]

63. Lewis KN, Liao R, Guinn KM, Hickey MJ, Smith S, Behr MA, et al. Deletion of RD1 from Mycobacterium tuberculosis mimics bacille Calmette-Guerin attenuation. J Infect Dis. 2003; 187:117–23. [PubMed: 12508154]

Highlights

- Three ATPase domains in the ESX ATPases contribute differently to secretion
- ATP hydrolysis by the first ATPase domain is required for ESX secretion
- Linked and split ESX ATPases differ in their requirement for nucleotide binding

Abbreviations

ESX early secretory antigen target 6 kDa (ESAT-6) secretion

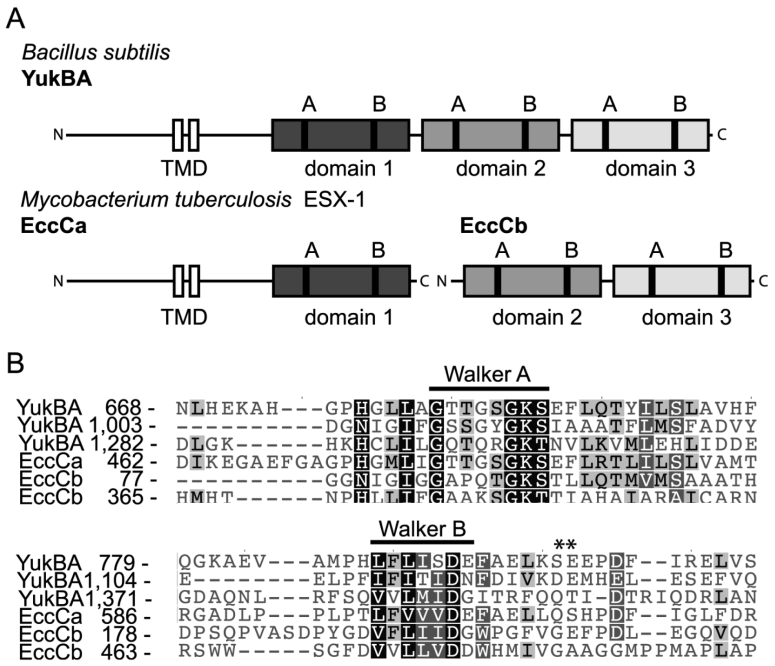

Figure 1. ATPases of the ESX secretion systems

(a) Protein domain structure of the ESX-1-encoded ATPases EccCa-EccCb of *M. tuberculosis* and the *yuk*-encoded ATPase YukBA of *B. subtilis*. Predicted FtsK-like ATPase domains are indicated as boxes of different shades of grey. The positions of Walker A and B motifs of each ATPase domain are indicated. (b) Excerpts of the alignment of all six ATPase domains of the YukBA, EccCa, and EccCa ATPases showing conserved Walker motifs. The additional “DE” site that was tested for the second YukBA domain is marked with double asterisk (**) above the alignment.

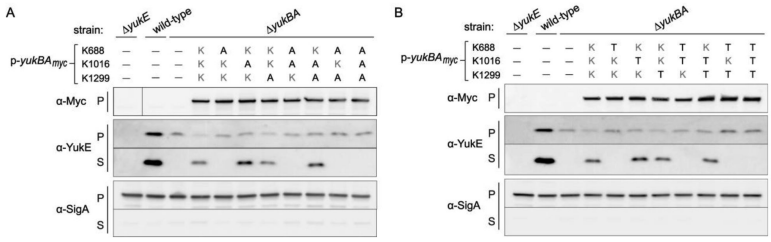

Figure 2. Walker A motif of the first ATPase domain of YukBA is required for substrate secretion

(a) and (b) Immunoblot analysis of cell pellet (P) and culture supernatant (S) of indicated strains grown in LB medium. Each *yukBA* Walker A motif mutant was C-terminally *myc*-tagged, fused to an IPTG- inducible promoter ($P_{hyperspank}$), and inserted in single copy at a nonessential locus in the chromosome of the Δ*yukBA* strain. A dash (−) indicates a strain lacking an integrated copy of *yukBA*. Strains expressing *yukBA* K to A (a) and K to T (b) Walker A motif mutants were assayed for YukE secretion. Reduced P and S samples were separated on an SDS-PAGE gel and analyzed by immunoblot with YukE-specific antibodies (antibody specificity is demonstrated by the Δ*yukE* strain). An α-Myc antibody was used to verify expression of the YukBA complementing constructs; an antibody to SigmaA was used as a lysis and loading control.

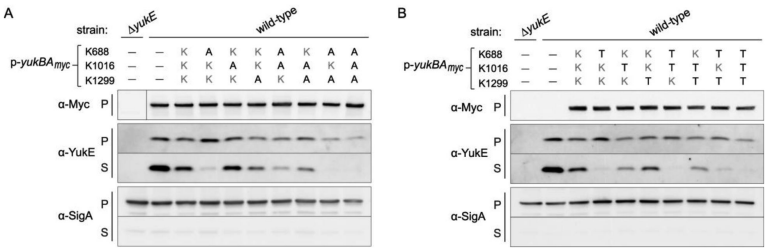

Figure 3. YukBA Walker A motif mutants might interfere with the function of the wild-type YukBA

(a) and (b) Immunoblot analysis of cell pellet (P) and culture supernatant (S) of indicated strains grown in LB medium. Each *yukBA* ATPase mutant gene was C-terminally *myc*-tagged, fused to an IPTG- inducible promoter ($P_{hyperspank}$), and inserted in single copy at a nonessential locus in the chromosome of an otherwise wild-type strain. A dash (−) indicates a strain lacking an integrated copy of *yukBA*. Strains expressing *yukBA* K to A (a) and K to T (b) ATPase mutants were assayed for YukE secretion. Reduced P and S samples were separated on an SDS-PAGE gel and analyzed by immunoblot with YukE-specific antibodies (antibody specificity is demonstrated by the Δ*yukE* strain). An α-Myc antibody was used to verify expression of the YukBA complementing constructs, and an antibody to SigmaA was used as a lysis and loading control.

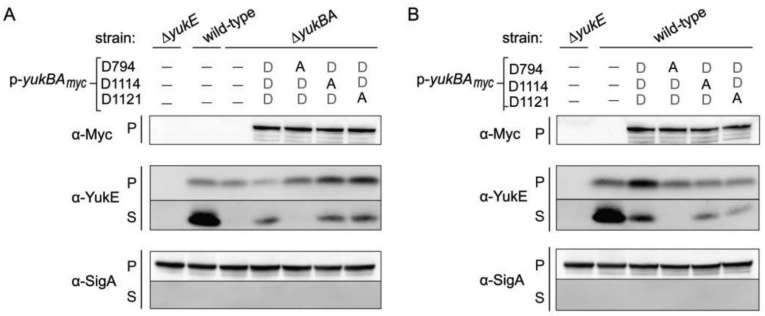

Figure 4. ATP hydrolysis in the first ATPase domain is required for secretion

(a) Immunoblot analysis of cell pellet (P) and culture supernatant (S) of indicated strains grown in LB medium. Each *yukBA* ATPase mutant gene was C-terminally *myc*-tagged, fused to an IPTG- inducible promoter ($P_{hyperspank}$), and inserted in single copy at a nonessential locus in the chromosome of an otherwise wild-type strain. A dash (-) indicates a strain lacking an integrated copy of *yukBA*. Strains expressing Walker B YukBA mutants were assayed for YukE secretion. Reduced P and S samples were separated on an SDS-PAGE gel and analyzed by immunoblot with YukE-specific antibodies. An α-Myc antibody was used to verify expression of the YukBA complementing constructs, and an antibody to SigmaA was used as a lysis and loading control. (b) Same as in (a) Walker B mutants of YukBA were expressed in wild-type *yuk* operon background. These strains were used to test the effects of the presence of two different YukBA copies on YukE secretion.

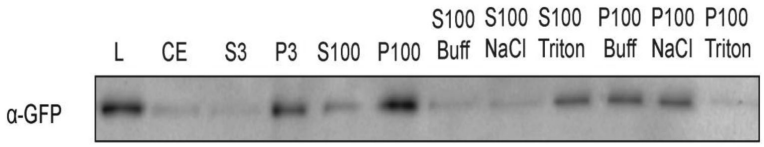

Figure 5. YukBA is integral membrane protein

Biochemical fractionation of YukBA-GFP was conducted to assess whether YukBA-GFP was a membrane-associated or integral membrane protein. YukBA-GFP has a predicted molecular weight of 199 kDa consistent with the species detected with GFP-specific antibodies. Soluble (S3) and insoluble (P3) protein fractions separated by centrifugation at 3,000 × g; soluble (S100) and insoluble (P100) fractions that were separated from the S3 fraction by centrifugation at 100,000 × g. The P100 fraction was further separated by incubation with either buffer, 1M NaCl, or 10% Triton detergent with subsequent spin at 100,000 × g to collect the soluble (S100) and insoluble (P100) fractions. YukBA elutes from the membrane P100 fraction only upon membrane solubilization with Triton that is indicative of an integral membrane protein.

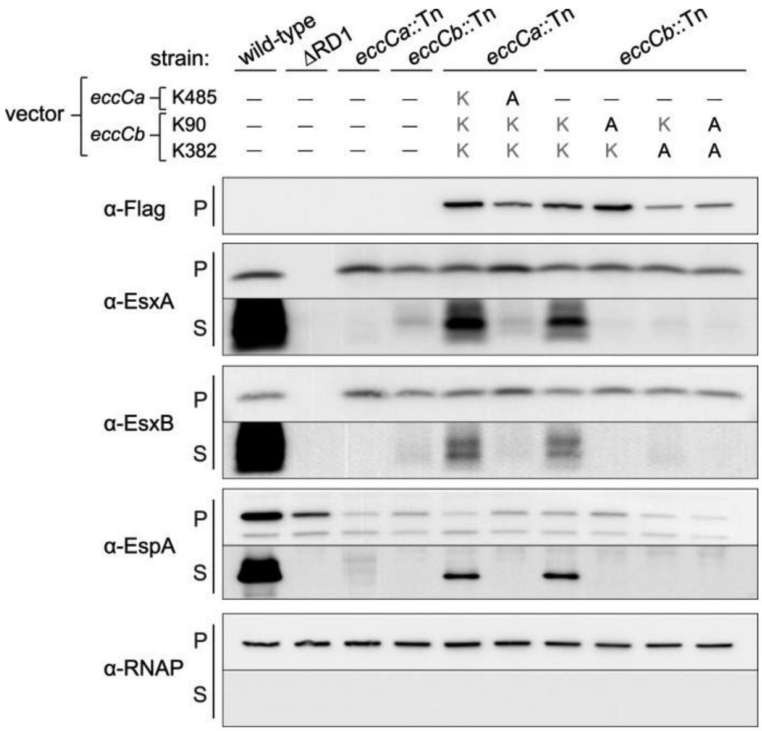

Figure 6. Each ATPase domain of the split EccCa-EccCb ATPase is required for secretion

Immunolot analysis of cell pellet (P) and culture supernatant (S) of the indicated strains grown in Sauton's medium. Each *eccCa* or *eccCb* Walker A motif mutant construct was episomally expressed in the corresponding *eccCa*::Tn or *eccCb*::Tn strain. A dash (-) indicates expression of an empty vector control. In each complementing construct, *eccCb* was C-terminally *flag*-tagged. Samples were separated by SDS-PAGE under reducing conditions and analyzed for ESX-1 secretion using antibodies specific to the ESX-1 substrates EsxA, EsxB, and EspA. An ESX-1 deletion mutant, ΔRD1 [63], was used as a negative control; an antibody to *E. coli* RNAP was used as a lysis and loading control. Data are representative of at least three independent experiments.

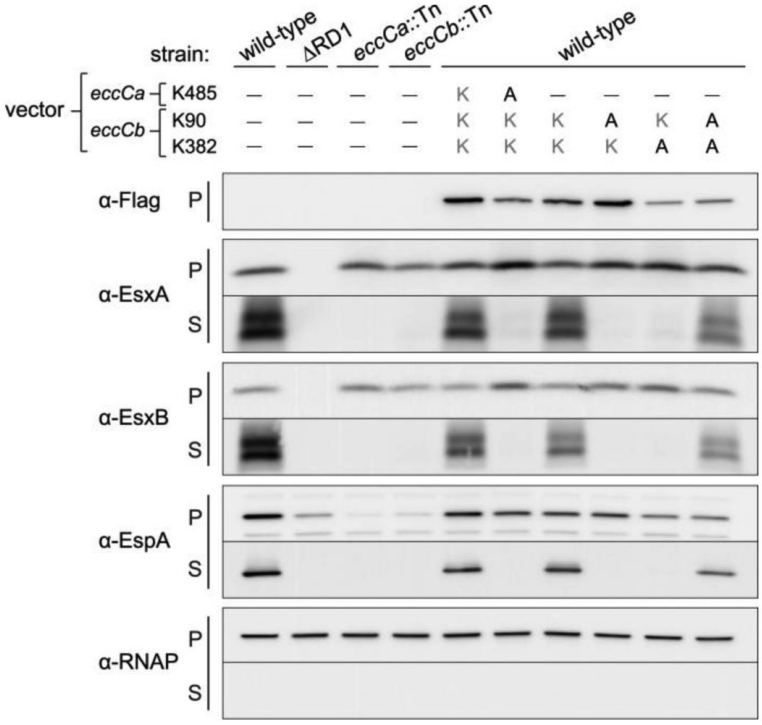

Figure 7. Some mutations in the ATPase domains of EccCa-EccCb exhibit a dominant negative effect on secretion

Immunoblot analysis of cell pellet (P) and culture supernatant (S) of the indicated strains grown in Sauton's medium. Each *eccCa* or *eccCb* Walker A motif mutant construct was episomally expressed in wild-type H37Rv. A dash (−) indicates expression of an empty vector control. In each complementing construct, *eccCb* was C-terminally *flag*-tagged. Samples were separated by SDS-PAGE under reducing conditions and analyzed for ESX-1 secretion using antibodies specific to the ESX-1 substrates EsxA, EsxB, and EspA. An ESX-1 deletion mutant, ΔRD1 [63], was used as a negative control; an antibody to *E. coli* RNAP was used as a lysis and loading control. Data are representative of at least three independent experiments.

Table 1

B. subtilis strains used in this study

Strain	Genotype	Source, Reference
PY79	Prototrophic domesticated laboratory strain	[56]
bLH014	*yukE::cat*	This work
bLH015	*yukE::erm-Pyuk*	[22]
bLH110	*yukBA::erm-Pyuk*	[22]
bLH266	*amyE::Phyperspank-yukBA-myc (spec)*	This work
bLH267	*amyE::Phyperspank-yukBA^{K688A}-myc (spec)*	This work
bLH268	*amyE::Phyperspank-yukBA^{K1016A}-myc (spec)*	This work
bLH269	*amyE::Phyperspank-yukBA^{K1299A}-myc (spec)*	This work
bLH318	*amyE::Phyperspank-yukBA^{K688A,K1016A}-myc (spec)*	This work
bLH319	*amyE::Phyperspank-yukBA^{K688A,K1299A}-myc (spec)*	This work
bLH320	*amyE::Phyperspank-yukBA^{K1016A,K1299A}-myc (spec)*	This work
bLH321	*amyE::Phyperspank-yukBA^{K688A,K1016A,K1299A}-myc (spec)*	This work
bLH397	*amyE::Phyperspank-yukBA^{K688T}-myc (spec)*	This work
bLH398	*amyE::Phyperspank-yukBA^{K1016T}myc (spec)*	This work
bLH399	*amyE::Phyperspank-yukBA^{K1299T}-myc (spec)*	This work
bLH400	*amyE::Phyperspank-yukBA^{K688T,K1016T}-myc (spec)*	This work
bLH401	*amyE::Phyperspank-yukBA^{K1016T,K1299T}-myc (spec)*	This work
bLH402	*amyE::Phyperspank-yukBA^{K688T,K1299T}-myc (spec)*	This work
bLH403	*amyE::Phyperspank-yukBA^{K688T,K1016T,K1299T}-myc (spec)*	This work
bLH404	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA-myc (spec)*	[22]
bLH405	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA^{K688A}-myc (spec)*	This work
bLH406	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA^{K1016A}-myc (spec)*	This work
bLH407	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA^{K1299A}-myc (spec)*	This work
bLH408	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA^{K688A,K1016A}-myc (spec)*	This work
bLH409	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA^{K1016A,K1299A}-myc (spec)*	This work
bLH410	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA^{K688A,K1299A}-myc (spec)*	This work
bLH411	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA^{K688A,K1016A,K1299A}-myc (spec)*	This work
bLH412	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA^{K688T}-myc (spec)*	This work
bLH413	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA^{K1016T}myc (spec)*	This work
bLH414	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA^{K1299T}myc (spec)*	This work
bLH415	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA^{K688T,K1016T}-myc (spec)*	This work
bLH416	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA^{K1016T,K1299T}-myc (spec)*	This work
bLH417	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA^{K688T,K1299T}-myc (spec)*	This work
bLH418	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA^{K688T,K1016T,K1299T}-myc (spec)*	This work

Strain	Genotype	Source, Reference
bTS254	*amyE::Phyperspank-yukBA*^{D794A}*-myc (spec)*	This work
bTS255	*amyE::Phyperspank-yukBA*^{D1115A}*-myc (spec)*	This work
bTS256	*amyE::Phyperspank-yukBA*^{D1121A}*-myc (spec)*	This work
bTS257	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA*^{D794A}*-myc (spec)*	This work
bTS258	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA*^{D1115A}*-myc (spec)*	This work
bTS259	*yukBA::erm-Pyuk; amyE::Phyperspank-yukBA*^{D1121A}*-myc (spec)*	This work
bLH001	Ω *yukBA-gfp (spec)*	This work

Table 2

M. tuberculosis strains used in this study

Strain	Genotype	Source, Reference
H37Rv	Wild-type laboratory strain	ATCC25618
ΔRD1	H37Rv::ΔRD1	[61]
eccCa::Tn	H37Rv::Tn3870	[18]
eccCb::Tn	H37Rv::Tn3871	[18]
H37RV-1	pTETSG (empty)	This work
eccCa::Tn-1	*eccCa*::Tn; pTETSG (empty)	This work
eccCb::Tn-1	*eccCb*::Tn; pTETSG (empty)	This work
H37Rv-57	pTETSG-*eccCb*^{K90A}-*flag*	This work
eccCb::Tn-57	*eccCb*::Tn; pTETSG-*eccCb*^{K90A}-*flag*	This work
H37Rv-58	pTETSG-*eccCb*^{K382A}-*flag*	This work
eccCb::Tn-58	*eccCb*::Tn; pTETSG-*eccCb*^{K382A}-*flag*	This work
H37Rv-59	pTETSG-*eccCb*$^{K90A, K382A}$-*flag*	This work
eccCb::Tn-59	*eccCb*::Tn; pTETSG- *eccCb*$^{K90A, K382A}$-*flag*	This work
H37RV-61	pTETSG-*eccCb-flag*	This work
eccCb::Tn-61	*eccCb*::Tn; pTETSG-*eccCb-flag*	This work
H37Rv-105	pTETSG-*eccCa-eccCb-flag*	This work
eccCa::Tn-105	*eccCa*::Tn; pTETSG-*eccCa-eccCb-flag*	This work
H37Rv-106	pTETSG-*eccCa*^{K485A}-*eccCb-flag*	This work
eccCa::Tn-106	*eccCa*::Tn; pTETSG-*eccCa*^{K485A}-*eccCb-flag*	This work

Table 3

B. subtilis plasmids used in this study

Plasmid	Description	Source
pLH016	*yukEr::cat*	This work
pLH018	*yukE::erm-Pyuk*	This work
pLH026	*yukBA::erm-Pyuk*	This work
pLH042	*amyE::Phyperspank-yukBA-myc (spec)*	This work
pLH054	*His6-SUMO-YukE*	This work
pTLR235	*amyE::Phyperspank-yukBA^{K688A}-myc (spec)*	This work
pTLR236	*amyE::Phyperspank-yukBA^{K1016A}-myc (spec)*	This work
pTLR237	*amyE::Phyperspank-yukBA^{K1299A}-myc (spec)*	This work
pTLR245	*amyE::Phyperspank-yukBA^{K688A,K1016A}-myc (spec)*	This work
pTLR246	*amyE::Phyperspank-yukBA^{K1016A,K1299A}-myc (spec)*	This work
pTLR247	*amyE::Phyperspank-yukBA^{K688A,K1299A}-myc (spec)*	This work
pTLR248	*amyE::Phyperspank-yukBA^{K688A,K1016A,K1299A}-myc (spec)*	This work
pTLR252	*amyE::Phyperspank-yukBA^{K688T}-myc (spec)*	This work
pTLR253	*amyE::Phyperspank-yukBA^{K1016T}-myc (spec)*	This work
pTLR254	*amyE::Phyperspank-yukBA^{K1299T}-myc (spec)*	This work
pTLR255	*amyE::Phyperspank-yukBA^{K688T,K1016T}-myc (spec)*	This work
pTLR256	*amyE::Phyperspank-yukBA^{K1016T,K1299T}-myc (spec)*	This work
pTLR257	*amyE::Phyperspank-yukBA^{K688T,K1299T}-myc (spec)*	This work
pTLR258	*amyE::Phyperspank-yukBA^{K688T,K1016T,K1299T}-myc (spec)*	This work
pTS237	*amyE::Phyperspank-yukBA^{D794A}-myc (spec)*	This work
pTS238	*amyE::Phyperspank-yukBA^{D115A}-myc (spec)*	This work
pTS239	*amyE::Phyperspank-yukBA^{D1121A}-myc (spec)*	This work
pLH001	Ω *yukBA-gfp (spec)*	This work

Table 4

M. tuberculosis plasmids used in this study

Plasmid	Description	Source, Reference
pTETSG	Episomal expression vector constructed to express genes under the control of a tetracycline inducible mycobacterial promoter	[62]
pTLR089	pTETSG-*eccCb*^{K90A}-*flag*	This work
pTLR090	pTETSG-*eccCb*^{K382A}-*flag*	This work
pTLR091	pTETSG-*eccCb*^{K90A,K382}*A-flag*	This work
pTLR171	pTETSG-*eccCa-flag*	This work
pTLR172	pTETSG-*eccCb-flag*	This work
pTLR184	pTETSG-*eccCa-eccCb-flag*	This work
pTLR250	pTETSG-*eccCa*^{K485A}-*eccCb-flag*	This work